PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau EV889154197US

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/00 A1

(11) International Publication Number: WO 92/15671

(43) International Publication Date: 17 September 1992 (17.09.92)

(21) International Application Number: PCT/US92/01867

(22) International Filing Date: 9 March 1992 (09.03.92)

(30) Priority data:
666,809 8 March 1991 (08.03.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 666,809 (CIP)

(71) Applicant *(for all designated States except US)*: CYTOMED, INC. [US/US]; 840 Memorial Drive, Cambridge, MA 02139 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : KO, Jone, Long [US/US]; 115 Ellery Street, Cambridge, MA 02138 (US). IP, Stephen, H. [US/US]; 11 Singing Hills Circle, Sudbury, MA 01776 (US).

(74) Agent: OLSTEIN, Elliot, M.; Carella, Byrne, Bain, Gilfillan, Cecchi & Stewart , 6 Becker Farm Road, Roseland, NJ 07068 (US).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: SOLUBLE CD28 PROTEINS AND METHODS OF TREATMENT THEREWITH

(57) Abstract

A soluble compound selected from the group consisting of: soluble CD28 and soluble derivatives and analogues thereof, which is capable of binding to a B-7 protein. The compound preferably has one of the following amino acid sequences of amino acid residues 19 to 108, 19 to 140 or 19 to 151, of human CD28 protein or a soluble derivative or analogue thereof. Most preferably the amino acid sequences of soluble CD28 is in dimeric form. Also processes for the treatment or prevention of immunological diseases and rejection of transplanted cell and tissue. Specifically the prevention of graft versus host diseases in bone marrow transplantation.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

SOLUBLE CD28 PROTEINS AND METHODS OF TREATMENT THEREWITH

BACKGROUND

This invention relates to CD28 protein, which is associated with T-cell immune responses. More particularly, this invention relates to soluble CD28 protein fragments and treatment methods employing such fragments.

CD28 (also known as Tp44: Weiss, A.; Manager, B. and Imboden, J., J. Immunol., 137, 819-825, 1986; Aruffo, A. and Seed, B. Proc. Natl. Acad. Sci., USA, 84, 8573-8577, 1987) protein is a membrane-bound T-cell differentiation antigen which includes a transmembrane domain. It is expressed on a large subset of peripheral blood T-lymphocytes as a membrane protein of homodimer with a molecular weight of 44 kDa. It has been demonstrated to be involved in T-cell activation by antigen (Weiss, A., Manager, B. and Imboden, J., J. Immunol., 137, 819-825, 1986). The complete gene sequence encoding CD28 has been published by Aruffo, A. and Seed, B., Proc. Natl. Acad. Sci., USA, 84, 8573-8577, 1987.

A recent study has shown that the CD28 antigen, expressed in Chinese Hamster Ovary cells, mediated specific intercellular adhesion with human lymphoblastoid and leukemic B-cell lines and with activated primary murine B cells. The specific recognition by CD28 of the B-cell activation antigen B7 (also known as BB-1) represents a heterophilic interaction between members of immunoglobulin superfamily that may serve to regulate T-cell cytokine levels at sites of B-cell activation. The results of the study have led the researchers to conclude that CD28 mediated adhesion may play a role in maintaining or amplifying the immune response, rather than initiating it. (Linsley, P.S., Clark, E.A., Ledbetter, J.A., Proc. Natl. Acad. Sci. USA, 87, 5031-5035, 1990).

In order for T-cells to mount an immune response toward a given antigen, two types of interactions between T-cells and antigen presenting cells are required. The first interaction occurs between the T-cell antigen receptors of T-cells and the antigen histocompatibility molecule complexes of antigen presenting cells. The second interaction is also required and it has been suggested that CD28 molecule of T-cells and and the B-7 molecule of the antigen presenting cells may be providing the required second signal. However, no publication has demonstrated that a soluble human protein can be used to suppress this specific pathway. (Freeman, G.J., et al., J. Immunol. 143, 2714-2722, 1989).

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a molecule which will block antigen presenting cells and thereby inhibit specific T-cell activation namely, the interaction between the Biomolecule B7 and CD28. The B7 molecule is sometimes referred to in the art as the BB-1 molecule. Applicants have discovered that there are soluble compounds which bind to the B-7 molecule and inhibits T-cell activation. The preferred compound is a soluble fragment of CD28 or a soluble analogue or derivative thereof; and most preferably this compound is a dimeric form of a soluble fragment of CD28 or a soluble analogue or derivative thereof.

Another object of Applicant's invention is a method of producing soluble CD28 to ensure a higher yield of the dimeric form of soluble CD28.

The term "fragment", "derivative" or "analogue" as used herein means modifications of the compound sequence in which amino acid residues have been deleted, inserted, or substituted without essentially detracting from the properties of the original sequence, such properties including solubility and the

ability to bind B-7. The term "tissue" as used herein is intended to include whole organs. A soluble fragment of CD28 which binds to the B-7 molecule is sometimes referred to herein as "soluble CD28." Additionally, the term "animal" is used in its broadest sense includes mammals including humans.

DETAILED DESCRIPTION OF THE DRAWINGS

The figures depicted and described herein are intended to further illustrate the present invention and are not intended to limit the invention in any manner whatsoever.

FIGURE 1. CD28 cDNA sequence.

This cDNA sequence is derived from Aruffo, A and Seed, B., Proc. Natl. Acad. Sci. USA, 84, 8573-8577, 1987. It encompasses the entire protein coding region for CD28 gene, for nucleotide Nos. 100 to 762. The lines above ATG (Nos. 100-102) and TGA (Nos. 760-762) indicate that they serve as the initiation and the termination codons respective for the translation of CD28 gene. The two underlined sequence clusters, markered A and B, correspond respectively to the two 3' end primers M1T and C1T cited in the example section.

FIGURE 2. DNA sequences of T7, M1T and C1T primers (SEQ ID Nos. 7 and 8, respectively).

T7 primer is 20 nucleotides long and its sequence is derived from PG3N vector purchased from Promega, Madison, WI, USA. T7 primer is used as the 5' end primer to pair with any 3' end primers, for example M1T and C1T, in a PCR reaction for the procurement of truncated CD28 gene which would direct the production of soluble CD28 protein. M1T and C1T, both are 21 nucleotides long, are derived from FIG. 1, markered as cluster A and B respectively. M1T spans from nucleotide Nos. 556 to 570 and that of C1T from Nos. 538 to Nos. 552. There are additional six nucleotides, ATCATC, added to the 5' end of M1T and C1T, to serve as the translational termination condons.

FIGURE 3. The Construction of CD28 expression vector.

A. pG3N-CD28 vector was constructed by the ligation of Xbal-Pstl CD28 cDNA fragment (Aruffo, A and Seed, B, Proc. Natl. Acad. Sci. USA, 84, 8573-8577, 1987) to the Xbal-Pstl site of PGEM-3fz plasmid (sometimes referred to herein as pG3N; Promega, Madison, WI). Arrows indicate the transcriptional direction for T7, T3 and CD28 gene. The shaded area represents the transmembrane region of CD28 protein after expression.

B. pG3N-CD28 was used as the DNA template in a PCR reaction, using T7-M1T and T7-C1T primer pairs for the generation of M1T and C1T DNA fragments, respectively. Both CD28 gene fragments were then subcloned into the EcoR1 site of p9 vector (Wong, G.G., et al., Science 228, 810-815, 1985). The resulting expression vectors, p9-M1T and p9-C1T, were used to transfect COS-7 cells and CHO for the production of soluble CD28 proteins.

FIGURE 4. CD28 protein sequence.

The CD28 cDNA encodes a protein of 220 amino acids (aa) in length (SEQ ID NO: 1). it contains a signal peptide from aa Nos. 1 to 18 and is cleavaged during the maturation of CD28 protein. The N-terminal of the mature CD28 protein starts at ASN, aa No. 19 (markered with a thick bar above AAC, nucleotide Nos. 153 to 155). The underlined sequences, aa Nos. 153 to 179, is the transmembrane region of CD28 protein. For the production of soluble CD28 protein, for example the one derived from p9-C1T, the transmembrane region has been deleted. The broken lines above the DNA sequences, markered as A and B, indicate the location of M1T and C1T primers, respectively. Thus, as discussed in the example provided herein the CD28 protein derived from P9-M1T has a length of 139aa (aa Nos. 19 to 157) (SEQ ID NO:9) which is not soluble CD28 and that of p9-C1T is 133aa long (aa Nos. 19 to 151) (SEQ ID NO:2) which is soluble CD28.

FIGURE 5.

Shows the entire CD28 gene construct and the truncated forms C3T, C2T, and C1T, as well as a chart identifying whether the truncated forms of CD28 are soluble, are dimeric and induce T-cell anergy.

FIGURE 6.

A. The DNA sequence of Primers, P1, P2, P3, and P4 used in PCR and their respective direction.

B. The DNA sequence for the linkage between C1T and PAP.

FIGURE 7.

Shows the construction of the C1PAP expression vector.

The CD28 gene segment C1T is generated through PCR using P1 and P2, the gene segment C1T is then treated with XhoI and XbaI before being subcloned into expression vector pBJ-neo. The resulting vector C1P was used for patching of gene segment PAP at the 3' end of C1T gene segment, resulting in the construction of the C1PAP expression vector.

FIGURE 8.

Shows the production of soluble Dimeric CD28 beginning with C1T and HPAP which are independently used in PCR and their ligation into pBJ-neo expression vector to form C1PAP. C1PAP is then transfected in CHO cells, amplified, subcloned, cell sorted and shaved from the cell membrane to yield a soluble dimeric form of CD28.

DETAILED DESCRIPTION OF THE INVENTION

Applicants have found that during antigen presentation by antigen presenting cells to T-cells, blocking the interaction between CD28 and B-7, inhibits T-cell activation (see examples cited herein pages 6 through 19). Such inactivity of T-cells toward antigens is valuable for certain clinical usages in treating diseases. Moreover, Applicant has found that soluble compounds which bind to B-7 can produce such inactivity.

Although the scope of the present invention is not intended to be limited to any theoretical reasoning, there are two theories which may explain the effect of soluble CD28 on T-cell activation.

I. That soluble CD28 or derivatives or analogues thereof which bind to the B-7 cell and thereby block the initial signal from the B-7 cell to the CD28 protein receptor, will permanently inactivate the T-cell's ability to recognize the foreign antigen, whereby a single treatment with soluble CD28 or derivatives or analogues thereof will permanently inhibit the T-cell response.

II. In the case of Autoimmune diseases where the T-cell has recognized the foreign antigen, it is believed possible to attentuate the T-cell response to prevent higher levels of T-cell activation. This would involve chronic treatment with soluble CD28 to maintain the attenuation of the T-cell by blocking the B-7 cell from signaling the CD28 protein.

Therefore, the soluble peptide or protein of the present invention can be useful for certain clinical applications. Applicants provide herein examples of the possible clinical uses for their invention, however, these uses are presented for illustrative purposes and are not intended to limit the application of this invention. For example: the treatment of autoimmune disease, immunosuppression diseases, T-cell mediated immunosuppression, rejection of transplanted cell and tissue, and specifically preventing or alleviating graft versus host disease in bone marrow transplants.

In accordance with an aspect of the present invention, there is provided a soluble compound capable of binding to B-7. In preferred embodiment of the present invention, there is provided soluble CD28 or derivatives or analogues thereof which are capable of binding to B-7. The procurement of a truncated CD28 gene, which directs the production of such a soluble CD28 or derivatives or analogues thereof, utilizes a 3' end primer in a

Polymerase Chain Reaction (PCR) as described in the examples provided herein. Thus, the 3' end primer which spans from nucleotide Nos. 538-552 derives the sequence for a preferred embodiment of soluble CD28 having the amino acid sequence of amino acid residue Nos. 19 to 151 (C1T) (SEQ ID NO. 2) of human CD28 protein or a soluble derivative or analogue thereof. The above-mentioned sequence C1T is a human CD28 molecule in which the transmembrane region (amino acid residue Nos. 153 to 179) has been deleted from the full-length human CD28 molecule. The resulting peptide or protein, encompassing amino acid residue Nos. 19 to 151, is a soluble CD28 molecule. The derivation of this sequence is described in detail in the examples provided herein.

In another embodiment of the present invention the 3' end primer which spans from Nucleotides Nos. 409-425 derives the sequence for a soluble CD28 having an amino acid sequence of amino acid residues Nos. 19-108 (C2T) (SEQ ID NO. 4) of human CD28 protein or a soluble derivative or analogue thereof.

Another embodiment of the present invention the 3' end primer which spans from Nucleotides Nos. 502-529 derives the sequence for a soluble CD28 having an amino acid sequence of amino acid residues Nos. 19-140 (C3T) (SEQ ID NO. 3) of human CD28 protein or a soluble derivative or analogue thereof.

In accordance with another aspect of the present invention, there is provided DNA which encodes soluble compounds capable of binding to B-7. Preferably, the soluble compounds have the amino acid sequence of amino acid residue Nos. 19 to 151 of human CD28 protein, or soluble derivatives or analogues of any of the sequences, as hereinabove described. It is to be understood that the DNA which encodes the soluble CD28 may be modified by deletion, insertion, or substitution of nucleotides to encode fragments, derivatives or analogues of the CD28 amino acid sequence hereinabove described, said fragments, derivatives or

analogues having the same properties as the unmodified CD28 amino acid sequence, including the ability to bind B-7.

The DNA which encodes the soluble compounds hereinabove described may be prepared by techniques known to those skilled in the art, such techniques including recombinant DNA techniques, or the DNA may be chemically synthesized. The preparation of C1T by these methods will inherently yield soluble CD28 of both monomeric and dimeric forms. It is the dimeric form of soluble CD28 which is responsible for inducing T-cell anergy. (See example I which yielded 90% monomeric form, 10% dimeric form.)

In accordance with another aspect of the present invention, there is provided an expression vector which includes DNA encoding for the soluble compounds hereinabove described.

In one embodiment, the expression vector may be a eukaryotic expression vector.

The following are examples for expression vectors which may be expressed as a eukaryotic expression vector:

pMSG uses the promoter from mouse mammary tumor virus long terminal repeat (MMTV). The suitable hosts for pMSG are Chinese hamster ovary cell, Hela cell and mouse Lkt negative cells (Lee, F., et al., Nature 294, 228-232, 1981).

pSVL uses SV40 late promoter and its suitable host cell is COS for high level transient expression (Sprague, J., et al., J. Virol. 45, 773-781, 1983; Gempleton, D. and Eckhart, W., Mol. Cell Biol. 4, 817-821, 1984).

pRSV uses Rous Sarcoma Virus promoter and its suitable hosts are mouse fibroblast cell, lymphoblastoid cell and COS cell (Gorman, C., Padmanabhan, R. and Howard, B.H., Science 221, 551-553, 1983).

pBPV is a DNA viral vector derived from bovine papilloma virus and its suitable for the stable expression in mouse mammary tumor cell, C127 (Zin, K., DiMaio, D. and Maniatis, T., Cell 34, 865-879, 1983; Sarver, N., et al., Mol. Cell Biol. 1, 486-496,

1981; Sarver, N., Byrne, J.C. and Howley, P.M., Proc. Natl. Acad. Sci., USA, 79. 7147-7151, 1982; Law, M.F., Byrne, J.C. and Howley, P.M., Mol. Cell Biol. 3, 2110-2115, 1983).

Baculovirus expression vector is suitable for the stable expression in insert cell, sf9 (Luckow, V.A. and Summers, M.D., Bio. Technology 6, 47-55, 1988; Miller, L.K., Ann Rev Microbiology 42, 177-199, 1988).

In another embodiment, the expression vector may be a prokaryotic vector.

The following expression vectors may be expressed as procaryotic expression vector:

pOX expression series uses oxygen-dependent promoter of vireoscilla hemoglobin gen in the E. Coli system (Khosla, G., et al., Bio. Technology 8, 554-558, 1990).

pPL expression series use the strong PL promoter of Lambda phase (Reed, R.R., Cell 25, 713-719, 1981; Simatake, H.Z. and Rosenberg, M., Nature 292, 128-132, 1981; Mott, J.D., et al., Proc. Natl. Acad. Sci. USA, 82, 88-92, 1985).

pKK223-3 uses a hybrid promoter derived from the fusion between to promoters of tryptophan and lactose operons of E. Coli origins (Brosius, J. and Holy, A., Proc. Natl. Acad. Sci., USA, 81, 6929-6933, 1984).

The expression vector may be employed to transform cells which will produce the soluble compounds. Examples of cells which may be transformed with the expression vector include Chinese hamster ovary cells, lymphoblastoid cell, (M. Okamoto, et al., Bio. Technol. 8, 550-553, 1990) and C127 mouse mammary tumor cell (J. Virol. 26, 291-298, 1978; Virology 103:369-375, 1980). The transformed cells, which express the soluble compounds may be employed in an in vitro expression system to produce soluble CD28 molecules.

Since as stated previously it is the dimeric form of soluble CD28 which is responsible for the T-cell inactivation, it would

therefore be advantageous to produce the dimeric form in greater amounts than that inherently yielded by the preparation of C1T by known techniques.

Therefore, another embodiment of the present invention provides a method for more efficiently producing and isolating the dimeric form of soluble CD28. The method involves the independent treatment of both (i) CD28-pg 3N vector with PCR and primers P1 and P2 to yield C1T (truncated form of soluble CD28) and (ii) human placental alkaline phosphatase (HPAP) with PCR and primers P3 and P4 to yield PAP (having 49 amino acids). The C1T and PAP are then ligated into an expression vector, which results in the C1T sequence being linked to the PAP sequence (of which only 18 amino acids remain) to form C1PAP. The last of the 18 amino acids of the PAP portion of C1PAP is arginine, which is available for the addition the phosphatidylinocitol glycan (pI-G). The pI-G acts as an anchor to hold the C1PAP to the cell membrane. Once on the cell membrane the C1T will be expressed as a dimeric recombinant soluble CD28 as a membrane protein. The dimeric protein can then be recovered from the membrane after treatment with Phospholipase C. After removal from the cell membrane the soluble CD28 will remain in its dimeric form.

The ability of soluble CD28 to form a dimeric is believed to occur through the Cys1, cystine residue (figure 5) the formation through Cys1 involves the crosslinking such formation of the disulfide bond in the dimeric CD28. it is also believed that the production of the dimeric form of soluble CD28 is enhanced by the anchoring of the C1T (soluble CD28) to the cell membrane.

The presence of Cys2 and Cys3, cystine residues (figure 5) are believed to be responsible for the bioactivity of the dimeric soluble CD28 compound. Although it is applicants' belief that other amino acids may be substituted for Cys2 and/or Cys3 while maintaining the bioreactivity of the compound.

In accordance with another aspect of the present invention, there is provided a process for the treatment of donor bone marrow cells to eliminate the potential for graft versus host disease in a bone marrow transplant patient. The process comprises administering ex vivo to the bone marrow of a donor of a bone marrow transplant an effective amount of soluble CD28 or soluble derivative or analogue thereof which is the dimeric form. Preferably, the process will involve perfusing, mixing or treating from about 100cc to about 1000cc of donor's bone marrow cells with at least 1cc of the dimeric form of soluble CD28, in a suitable pharmaceutical media for a period of time no longer than 24 hours, and then combining the recipient's bone marrow cells with that of the donor's cells. Then a conventional bone marrow cell reintroduction procedure can be used to infuse the treated cells into the recipient.

More specifically, the process as described involves perfusing, mixing or treating the donor's bone marrow cells with from about 1cc to about 50cc of soluble CD28, preferably CD28 is in dimeric form, for a period of time from about 10 minutes to about 4 hours. Pharmaceutical media as mentioned above includes all media suitable to sustain living cells, such media should contain all the necessary nutrients and trace elements for normal growth of the bone marrow cells.

Another aspect of the present invention is a process for treating T-cell mediated immunosuppression in an animal which comprises administering to the animal an effective amount of a dimeric form of soluble CD28 or a soluble analogue or derivative thereof. Preferably, the process will involve administering to the animal an intravenous injection of from about 2ug to about 2mg per kg of body weight of the animal of the a dimeric form of soluble CD28 or a soluble analogue or derivative thereof compound.

Another aspect of this invention is provided a process for treating automimmune disease in an animal which comprises administering to said animal an effective amount of a dimeric form of soluble CD28 or derivatives or analogues thereof. A preferred embodiment of the process involves a chronic intravenous administration of from about 2ug to about 2mg per kg of body weight of the animal of the soluble CD28 or soluble derivative or analogue thereof.

Another aspect of the present invention is a process for preventing rejection of transplanted cell and tissue in a host comprising administering to the host an effective amount of soluble CD28 or a soluble analogue or derivative thereof. Preferably, the process will involve administering to the host an intravenous injection of from about 2ug to about 2mg per kg of body weight of the host of the dimeric form of soluble CD28 or a soluble analogue or derviative thereof.

In another embodiment the effective amount of dimeric form of soluble CD28 or a soluble analogue or derivative thereof, is administered as part of a pharmaceutical composition in conjunction with an acceptable pharmaceutical carrier. Acceptble pharmaceutical carriers include but are not limited to non-toxic buffers, fillers, and physiological saline solution.

Due to their binding ability soluble CD28 or a soluble analogue or derivative thereof can be used in known procedures for selecting, identifying or assaying the presence of cells containing B-7 protein in sample, e.g., a body fluid. The CD28 of the present invention can for example, be attached or bound to a solid support, such as latex beads, a column, etc. which are then contacted with a sample containing cells having B-7 protein to bind such cells to the soluble CD28 on the support, preferably the dimeric form of soluble CD28. Therefore, soluble CD28 can be used in place of a monoclonal antibody to locate B-7 cells for

identification, selection, isolation and purification of antigen presenting cells containing B-7 protein, using known techniques.

The invention will now be described with respect to the following example; however, the scope of the present invention is not intended to be limited thereby.

Example I

In the following example, all restriction enzymes and custom synthesized oligo-primers were obtained from New England Biolabs, Beverly, Massachusetts. pGEM-3fz plasmid (sometimes referred to herein as pG3N) and T7 promoter primer were purchased from Promega, Madison, Wisconsin. Taq polymerases were obtained from New England Biolabs, Promega, and Cetus, of Norwalk, Connecticut.

COS-7 (ATCC No. CRL1651) cells were routinely maintained in Dulbecco's modified Eagle medium supplemented with 2.5% fetal bovine serum, 1% GMS-X (Gifco, Gaithersburg, Md.), 10 mM glutamin, 20 mM HEPES, 100 µg/ml streptomycin, and 100 units/ml penicillin.

A. Construction of pG3N-CD28 plasmid

The DNA sequence encoding for full-length CD28 is shown in Figure 1. The entire CD28 cDNA insert was excised from its original plasmid vector (Aruffo, A. and Seed, B. Proc. Natl. Acad. Sci. 84, 8573-8577, 1987) with XbaI and PstI restriction enzymes and subcloned into pG3N vector, which was also linearized with the same pair of enzymes. The resulting plasmid, pG3N-CD28 (Figure 3), has the same transcriptional orientation as that of the T7 promoter, while the orientation of the T3 promoter is in the opposite direction.

B. Polymerase Chain Reaction (PCR).

To construct a CD28 expression vector that would produce soluble CD28 molecules in an expression system, it is necessary to eliminate the transmembrane region for secretion of the soluble molecules into culture supernatant in an in vitro expression system. A schematic of the construction of such a vector is shown in Figure 2. To achieve the production of soluble CD28, PCR is employed to generate a series of progressive deletions of nucleotides of the CD28 gene into the extracellular region, away from the transmembrane region.

The PCR reaction is carried out under conditions as described in S.J. Scharf, 1990 PCR Protocol: A guide to methods and applications; Ed. M.A. Innis, D.H. Gelfrand, J.J. Sninsky and J.J. White, Academic Press Inc., New York, New York pp. 89-91. In a typical 100 µl reaction, the reaction mixture contained 50 mM KCl, 10mM Tris-HCl, pH8.3, 1.5mM $MgCl_2$, 0.2 mM of all four deoxyribonucleotides, 0.001% gelatin, 1ng of linearized pG3N-CD28 template, 15 pmole of each of the paired primers, and 2.5 units of Taq polymerase. The amplification reaction was run for 25 cycles with each cycle comprised of incubation of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute. At the end of the last cycle, a further incubation at 72°C for an additional 15 minutes was allowed in order to complete the amplification reaction.

In the PCR reaction, a primer locates at the 5' end of the CD28 cDNA insert. The 5' end primer is paired with a variety of 3' end primers whose sequences are dictated by the exact truncation sites to be reconstructed.

A T7 promoter primer, a 20mer obtained from Promega, having the following sequence:

5'--TAA TAC AC TCA CTA TAG GG--3'

was employed as the universal 5' end primer for the construction of truncated CD28 gene fragments. DNA fragments amplified from a starting point of the T7 primer contain sequences derived from

the T7 promoter, several unique restriction sites and a CD28 gene sequence whose end is determined by the 3' end primer employed. The unique restriction sites are used to isolate expression vectors that had the CD28 gene fragment inserted in the correct orientation in accord with that of the adenovirus late major promoter (LMP) which would serve as the promoter for the CD28 insert.

The selection of 3' end primers is dictated by the sites where one desires the CD28 gene to be truncated. In general, the 3' end primers are 21 nucleotides long with 15 nucleotides derived from CD28 gene sequences and six extra nucleotides added at the 5' end that can serve as the termination codons for the translation of the truncated CD28 gene.

Two 3' end primers, known as pC1T and pM1T (two consecutive stop condons), were synthesized (New England Biolabs). pC1T spans nucleotide Nos. 535 to 552 of the CD28 gene, and pM1T spans nucleotide Nos. 556 to 573. Six nucleotides are added to one end of each of the two primers to serve as the termination codons (two stop condons) for the translation of the truncated CD28 gene. PCR amplification employing pC1T yields a truncated CD28 gene fragment (C1T) of 615 base pairs, and PCR amplification employing pM1T yields a truncated CD28 gene fragment (M1T) of 633 base pairs.

After amplification, the CD28 DNA fragments are further purified through phenol extraction, ethanol precipitation, and gel electrophoresis in 1% low melting point agarose. The DNA band containing the CD28 gene fragment is then excised from the gel, phenol extracted, and ethanol precipitated for subcloning into an expression vector.

C. Subcloning of CD28 gene fragments into expression vectors.

The C1T and M1T CD28 gene fragments each were subcloned into the EcoRI site of the p91023(B) vector (sometimes hereinafter referred to as p9, Wong, G.G., et al., Science 228, 810-815,

1985). p9 is a eukaryotic expression vector. The insertion of both CD28 gene fragments at the EcoRI site of the p9 vector rendered them subjected to the regulation of the adenoviral LMP promoter located upstream from the CD28 gene fragments. Two CD28 expression vectors, p9-C1T and p9-M1T were then isolated.

p9-C1T directs the synthesis of a truncated protein containing the entire extracellular domain of the native CD28 protein except for the proline residue immediately before the transmembrane region, while p9-M1T directs the synthesis of a truncated CD28 protein with the complete extracellular domain and also five amino acids of the transmembrane region. Both expressed proteins carry a signal peptide of 19 amino acids long which is cleaved after expression. Thus, the expressed p9-C1T and p9-M1T proteins contain 133 and 139 amino acids, respectively.

D. Transfection of COS-7 cells.

The day before transfection, COS-7 cells were subcultured in new dishes at a cell density which would give about 70 to 80% confluency the following day. Just before transfection, COS-7 cells were washed twice with opti-MEM medium (Gifco) without serum.

Lipofectin was used to introduce the expression vector (either p9-C1T or p9-M1T) according to the protocol recommended by the manufacturer Gifco., Gaithersburg, M.D. USA. Two 1.5ml aliquots of opti-MEM, each containing 5 to 10 µg of expression vector (p9-C1T or p9-M1T) and 20µg of lipofectin separately, were pooled and mixed through pipetting before being added to the washed COS-7 monolayer. Transfection was allowed to take place for 6 hours at 37°C in a CO_2 incubator. The transfection solution was then removed and replaced with 5ml of opti-MEM supplemented with 2.5% fetal bovine serum, 1% GMS-X, 100 µg/ml streptomycins and 100 u/ml penicillin.

The transfected COS-7 cells were then incubated further without disturbance at 37°C for 3 days. The culture supernatant was collected and briefly spun to separate out dead cells before conducting assays for the induction of inhibition T-cell activation.

E. Assay for inhibition of T-cell activation.

COS-7 cells transfected with the p9 vector without the truncated CD28 gene sequence, and lipofectin alone were employed as negative controls to assess whether they would have any effect on the biological activity in the assay.

Culture supernatants derived from COS-7 cells transfected with p9-C1T (soluble CD28) and p9-M1T were collected and tested for biological activity in an in vitro assay for the inhibition of T-cell activation, also known as T-cell anergy. Initial results indicated that culture supernatant of p9-C1T gave a 50% inhibition of T-cell activation as measured by the reduction of Interleukin 2 (IL-2) production of the target T-cells, while supernatants derived from p9-M1T and p9 (control) or lipofectin alone failed to display any inhibition of T-cell activation in the assay.

Thus, a CD28 which was not soluble (p9-M1T) did not inhibit T-cell activiation whereas soluble CD28 (p9-C1T) did inhibit T-cell activation. The p9-C1T soluble CD28 segment was approximately 90% monomeric and 10% dimeric, the dimeric portion of the soluble CD28 is responsible for the inhibition of T-cell activation.

Example II

Cell Culture:

CHO cell line are routinely maintained in Iscove's MEM (Gifco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gifco), 100U/ml of penicillin, and 100ug/ml of

stroptomycin. For drug selection and amplification of CHO transfectants, methotrexate(Sigma, St. Louis, MO) were added at various concentrations.

Polymerase Chain Reaction(PCR):

The condition for PCR has been described (4). Briefly, in a typical 100ul reaction, the reaction mixture contained 50mM KCl, 10mM Tris-HOl, PH 8.3, 1.5mM $MgCl_2$, 0.2mM of all four deoxyribonucleotides, 5ng of template, 45pmole of each of the paired primers and 5 units of Taq polymerase. The amplification reaction was run for 30 cycles with each cycle comprised of incubation at 94°C for 1 min., 55°C for 1 min, and 72°C for 1 min. At the end of the last cycle a further incubation at 72°C for additional 15 min. was allowed to finish up the amplification reaction.

Transfection of CHO cell line:

The day before the transfection procedure was to be performed CHO cell was subcultured in a new dish at a cell density that would give at about 20% confluency the following day. Just before transfection CHO cell was washed twice with opti-I MEM (Gifco) without serum.

Lipofectin(Gifco) was used to introduce vectors into CHO cell according to manufacturer's protocol. Briefly, two 1.5ml of opt-I MEM each contain 10 ug of vectors and 100ug of lipofectin separately were pooled and added to the washed CHO cell. The transfection was allowed to proceed for 6 hours at 37°C in a CO2 incubator. The transfection solution was then replaced with regular culture medium for CHO cell for three days before the addition of methotrexate.

A. Construction of C1PAP expression vector.

A CD28 gene segment, CIT (1), has been generated through polymerase chain reaction(PCR) using primer p1 and p2 (fig. 7). p1, 5'CCTCGAGCATGCTCAGGCTGCTCTTG-3', a 26 mer, was used as the protein containing the signal peptide at its N-terminal(amino acids; #1 to 18), which is cleaved off in mature protein, and an entire extracellular portion (aa# 19 to 151) except the proline residue right before the transmembrane region.

C1T gene segment was then treated with Xho I and Xba I before its subcloning into an expression vector pBJ-neo (5) that was also cleaved with the same set of restriction enzymes. The resulting vector C1P, was then used for the patching of a gene segment, PAP, at the 3' end of the C1T gene segment.

PAP is the end product of a PCR reaction using HPAP-SK plasmid and primers p3 and p4(fig.5). HPAP-SK was derived from a human placental alkaline phosphatase (HPAP) cDNA clone (2,6), from nucleotide # 1475 to #1618, was cloned into bluescribe sk plasmid. This gene segment can encode for the last 49 amino acids (aa #467 to 513), the C-terminal, of the PLAP protein.

Primer p3, 5'-AGTCTAGATGCCTGGAGCCCTACACC-3', a 26 mer was used as the forward primer, while p4, 5'-TTATCAGGGAGCAGTGGCCGTCTC-3', a 24 mer as the reverse primer for the generation of a PAP. PAP spans the same region as that of HPAP and also acquire a Xba I at its '5 end and a flushed end at 3' end. It was cleaved with Xba I and then patched to the Xba I site right after C1T, and its flushed end is joined to the Not I site of the C1P vector. A new expression vector was thus constructed, designated as C1PAP.

B. The expression and production of soluble dimeric CD 28 protein.

C1PAP expression vector has been co-transfected with pSV2-DHFR vector into CHO cell line. The transfectants have been subjected to the selection of methotrexate at increasing concentrations. Those transfectants were then sorted through the staining of monoclonal antibody against CD28 and subcloned. Clones are maintained in culture medium containing 10uM of methotrexate.

C1PAP encoded for a protein contains 151 amino acids derived from CD28, two extra amino acids, serine and arginine, due to the strategy of vector construction and 49 amino acids from PAP (fig. 3). Eighteen out of 151 amino acids of CD28, locates at the very N-terminal, are signal peptide which will be trimmed off after its expression on the cell surface. Thirty of the 49 amino acids of PAP, locates at the very C-terminal, will be trimmed off and leaving 18 amino acids, the last amino acid being arginine, which is available for the addition of phosphatidyl-inositol glycan(pI-G)(7). It is through this pI-G anchor that the dimeric recombinant CD28 expressed as a membrane bound protein. Phsopholipase C can be used to recover the membrane bound CD28 from the cell surface with good efficiency.

Those transfectants have been shaved with phospholipase C and their supernatants examined. All shaved supernants contain soluble dimeric CD28 with an estimated mol. wt. of 40kd and 80kd in a reducing and non-reducing SDS-PAGE gel.

We believe the formation of dimeric recombinant CD28 generated in our lab. is through the cystine residue #1 (C1T) (Fig. 1) of CD 28. There are two observations support this

notion; (1.) human placental alkaline phosphatase is a non-covalently bound, dimeric protein(6); (2.) our experiment has demonstrated that cystine #1 is the candidate for the disulfide bond formation in the dimeric CD28.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

References:

1. A. Aruffo & B. Seed. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc. Natl. Acad. Sci. USA(1987) 48:8573-8577

2. S. Huet, H. Groux, B. Caillou, H. Valentin, A.-M. Prieur & A. Bernard. CD44 contributes to T cell activation. J. Immunol. (1989) 143: 798-801

3. P. S. Linsley, E. A. Clark & J. A. Ledbetter. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc. Natl. Acad. USA(1990) 87: 5031-5035

4. K. B. Mullis, F. A. Faloona, S. Sharf, R. Saiki, g. Horn & H. Erlich. Specificenzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol.(1986) 51: 263-273.

5. A. Y. Lin, B. Devaus, A. Green, C. Sagerstrom, J. G. Elliott & M. Davis. Expresion of T cell antigen receptor heterodimers in a lipid-linked form. Sciences (1990) 249: 677-679.

6. W. Kam, E. Clauser, Y.S. Kim, Y.W. Kan & W. J. Rutter. Cloning, sequencing, and chromosomal localization of human term placental alkaline phosphatase cDNA. Proc. Natl. Acad. Sci. USA(1985) 82: 8715-8719.

7. R. Micanovic, D. A. Bailey, L. Brink, L. Gerber, Y.-C. E. Pan, J. D. Hulmes & S. Udenfriend. Aspartic acid-484 of nascent placental alkaline phophatase condenses with a phosphatidylinositol gylcan to become the carboxyl terminus of the mature enzyme. Proc. Natl. Acad. Sci. USA (1988) 85: 1398-1402.

WHAT IS CLAIMED IS:

1. A soluble compound which binds to a B-7 protein, wherein the compound is selected from the group consisting of: soluble CD28, and soluble derivatives and analogues thereof.

2. The compound of Claim 1, wherein said soluble CD28 or soluble derivatives and analogues thereof is in dimeric form.

3. The compound of Claim 2 wherein said compound has an amino acid sequence of amino acid residue Nos. 19 to 151 of CD28 protein (SEQ ID NO:2), or a derivative or analogue of said protein.

4. A process for treating T-cell mediated immunosuppression in an animal which comprises:

administering to the animal an effective amount of the compound of Claim 2.

5. The process of Claim 3 wherein said effective amount further comprises an intravenous injection of from about 2ug to about 2mg per kg of body weight of said animal of said compound.

6. A process for preventing rejection of transplanted cell or tissue in a host comprising:

administering to the host an effective amount of the compound of Claim 2.

7. The process of Claim 6 wherein said effective amount further comprises an intravenous injection of from about 2ug to about 2mg per kg of body weight of said host of said compound.

8. A process for treating autoimmune disease in an animal which comprises:

administering to the animal an effective amount of the compound of Claim 2.

9. The process of Claim 8 wherein said effective amount further comprises chronic intravenous injection of from about 2ug to about 2mg per kg of body weight of said animal of said compound.

10. A process for preventing graft versus host disase in a bone marrow transplant which comprises:

perfusing, mixing, or treating ex vivo from about 100cc to about 1000cc of the bone marrow cells of a donor of a bone marrow transplant with at least 1cc of the compound of Claim 2 in a suitable pharmaceutical media, for a period of time not to exceed 24 hours,

combining the donor's bone marrow cells with a recipient's bone marrow cell, and

reintroducing said combination into the recipient.

11. The process of Claim 10, wherein said donor's bone marrow cells are perfused, mixed or treated with from about 1cc to about 50cc of said compound for from about 10 minutes to about 4 hours.

12. A process for preventing binding of a cell containing a B7 protein to a T-cell containing a CD28 receptor comprising:

contacting said cell containing a B7 protein with an effective amount of the compound of Claim 2.

13. A composition for preventing binding of a cell containing a B-7 protein to a T-cell containing a CD28 receptor comprising:

soluble CD28 or a soluble derivative or analogue thereof which binds to a B-7 protein and an acceptable pharmaceutical carrier.

14. A composition as in Claim 13, wherein said soluble CD28 or soluble derivative or analogue thereof is in dimeric form.

15. DNA encoding a compound selected from the group consisting of: soluble CD28 or soluble derivatives and analogues thereof, which is capable of binding to a B7 protein.

16. DNA encoding as in Claim 15, wherein said soluble CD28 or soluble derivatives and analogues thereof is in dimeric form.

17. The DNA of Claim 16 wherein said encoded compound has an amino acid sequence of amino acid residue Nos. 19 to 151 of

CD28 protein (SEQ ID NO:2) or a soluble derivative or analogue thereof.

18. An expression vector including the DNA of Claim 16.

19. A cell transformed with the expression vector of Claim 20.

20. An expression vector as in Claim 18 wherein, said expression vector is expressed as a eukaryotic or prokaryotic vector.

21. A cell as in Claim 19, wherein said cell is a mammalian, or bacterial cell.

FIG. 1

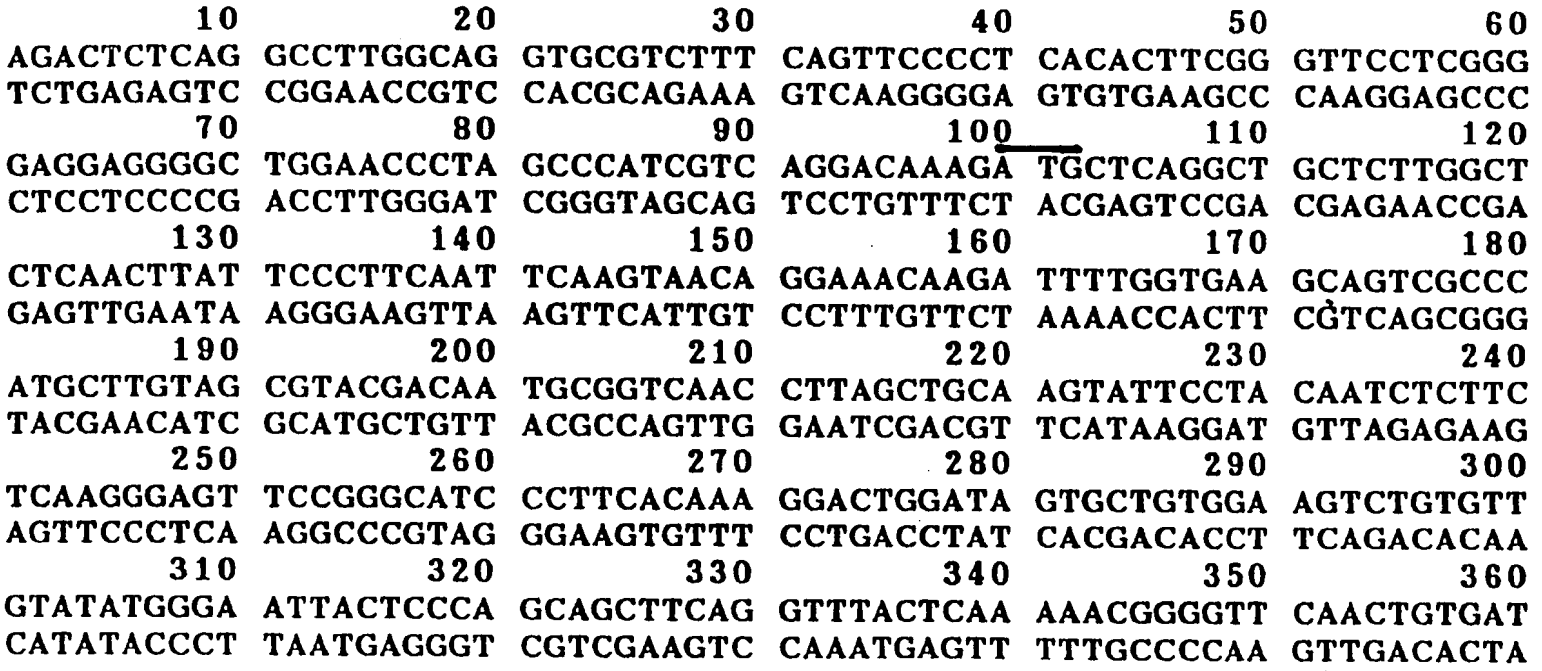

10 20 30 40 50 60
AGACTCTCAG GCCTTGGCAG GTGCGTCTTT CAGTTCCCCT CACACTTCGG GTTCCTCGGG
TCTGAGAGTC CGGAACCGTC CACGCAGAAA GTCAAGGGGA GTGTGAAGCC CAAGGAGCCC
70 80 90 100 110 120
GAGGAGGGGC TGGAACCCTA GCCCATCGTC AGGACAAAGA TGCTCAGGCT GCTCTTGGCT
CTCCTCCCCG ACCTTGGGAT CGGGTAGCAG TCCTGTTTCT ACGAGTCCGA CGAGAACCGA
130 140 150 160 170 180
CTCAACTTAT TCCCTTCAAT TCAAGTAACA GGAAACAAGA TTTTGGTGAA GCAGTCGCCC
GAGTTGAATA AGGGAAGTTA AGTTCATTGT CCTTTGTTCT AAAACCACTT CGTCAGCGGG
190 200 210 220 230 240
ATGCTTGTAG CGTACGACAA TGCGGTCAAC CTTAGCTGCA AGTATTCCTA CAATCTCTTC
TACGAACATC GCATGCTGTT ACGCCAGTTG GAATCGACGT TCATAAGGAT GTTAGAGAAG
250 260 270 280 290 300
TCAAGGGAGT TCCGGGCATC CCTTCACAAA GGACTGGATA GTGCTGTGGA AGTCTGTGTT
AGTTCCCTCA AGGCCCGTAG GGAAGTGTTT CCTGACCTAT CACGACACCT TCAGACACAA
310 320 330 340 350 360
GTATATGGGA ATTACTCCCA GCAGCTTCAG GTTTACTCAA AAACGGGGTT CAACTGTGAT
CATATACCCT TAATGAGGGT CGTCGAAGTC CAAATGAGTT TTTGCCCCAA GTTGACACTA

FIG. 1 CONT.

```
       370         380         390         400         410         420
GGGAAATTGG GCAATGAATC AGTGACATTC TACCTCCAGA ATTTGTATGT TAACCAAACA
CCCTTTAACC CGTTACTTAG TCACTGTAAG ATGGAGGTCT TAAACATACA ATTGGTTTGT
       430         440         450         460         470         480
GATATTTACT TCTGCAAAAT TGAAGTTATG TATCCTCCTC CTTACCTAGA CAATGAGAAG
CTATAAATGA AGACGTTTTA ACTTCAATAC ATAGGAGGAG GAATGGATCT GTTACTCTTC
       490         500         510         520         530         540
AGCAATGGAA CCATTATCCA TGTGAAAGGG AAACACCTTT GTCCAAGTCC CCTATTTCCC
TCGTTACCTT GGTAATAGGT ACACTTTCCC TTTGTGGAAA CAGGTTCAGG GGATAAAGGG
       550         560         570         580         590         600
GGACCTTCTA AGCCCTTTTG GGTGCTGGTG GTGGTTGGTG GAGTCCTGGC TTGCTATAGC
CCTGGAAGAT TCGGGAAAAC CCACGACCAC CACCAACCAC CTCAGGACCG AACGATATCG
       610         620         630         640         650         660
TTGCTAGTAA CAGTGGCCTT TATTATTTTC TGGGTGAGGA GTAAGAGGAG CAGGCTCCTG
AACGATCATT GTCACCGGAA ATAATAAAAG ACCCACTCCT CATTCTCCTC GTCCGAGGAC
       670         680         690         700         710         720
CACAGTGACT ACATGAACAT GACTCCCCGC CGCCCCGGGC CCACCCGCAA GCATTACCAG
GTGTCACTGA TGTACTTGTA CTGAGGGGCG GCGGGGCCCG GGTGGGCGTT CGTAATGGTC
       730         740         750         760
CCCTATGCCC CACCACGCGA CTTCGCAGCC TATCGCTCCT GA 3'
GGGATACGGG GTGGTGCGCT GAAGCGTCGG ATAGCGAGGA CT 5'
```

FIG. 2

A. 5' Primer:

T7: 5' --- TAA TAC GAC TCA TTA TAG GG --- 3'

B. 3' Primers:

a. M1T: 3' -- AAA ACC CAC GAC CAC ATC ATC -- 5'

b. C1T: 3' -- GGG CCT GGA AGA TTC ATC ATC -- 5'

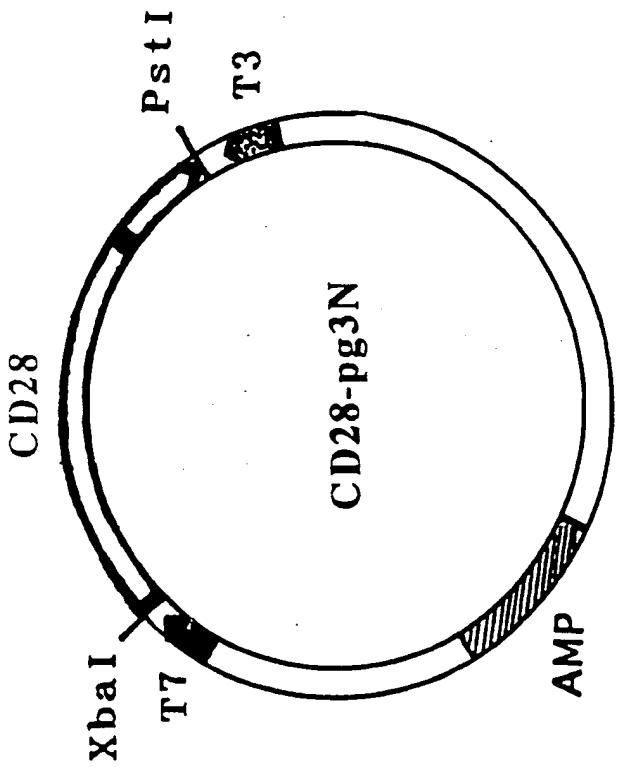


FIG. 3

FIG. 3 CONT.

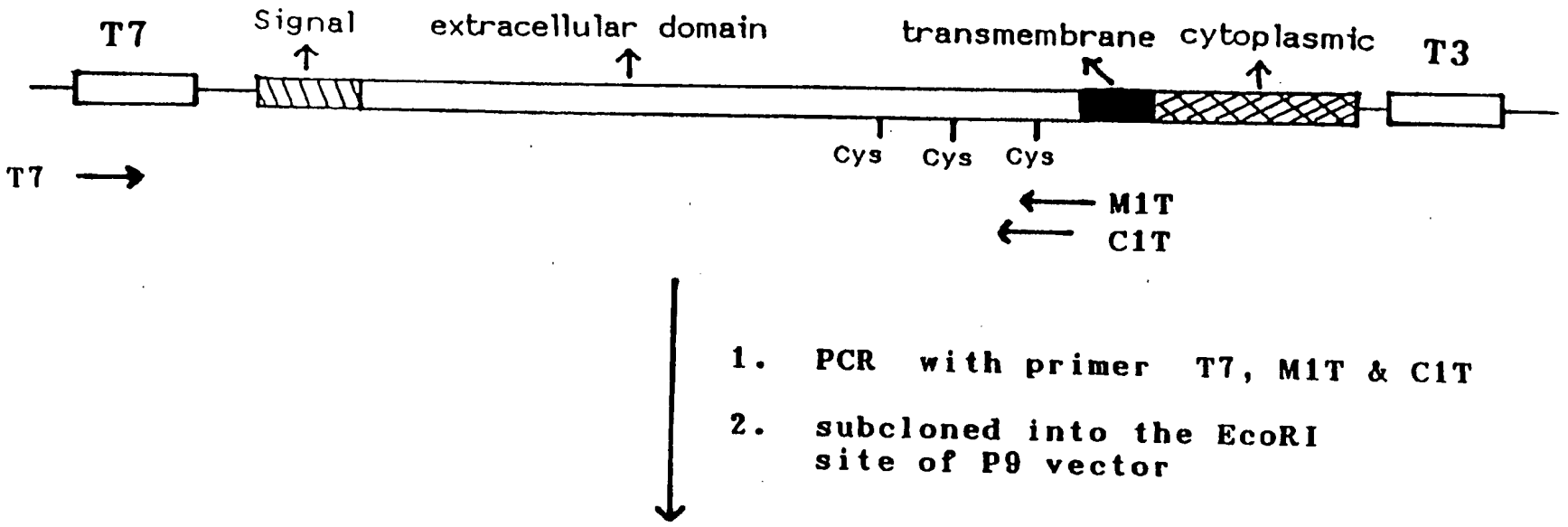
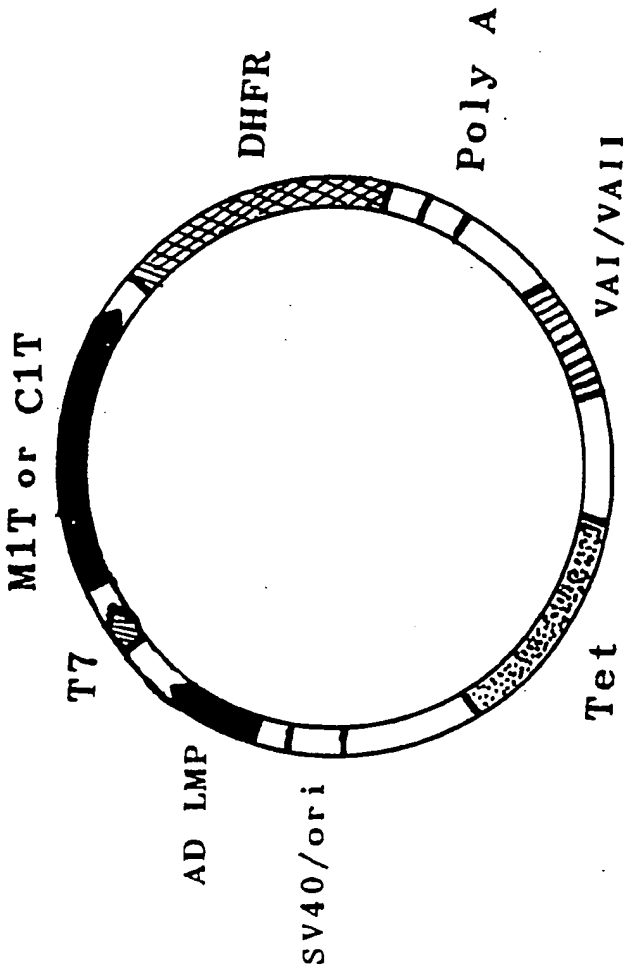

T7
Signal
extracellular domain
transmembrane
cytoplasmic
T3
Cys
Cys
Cys
T7
M1T
C1T
1. PCR with primer T7, M1T & C1T
2. subcloned into the EcoRI site of P9 vector

FIG. 3 CONT.



P9-M1T or P9-C1T

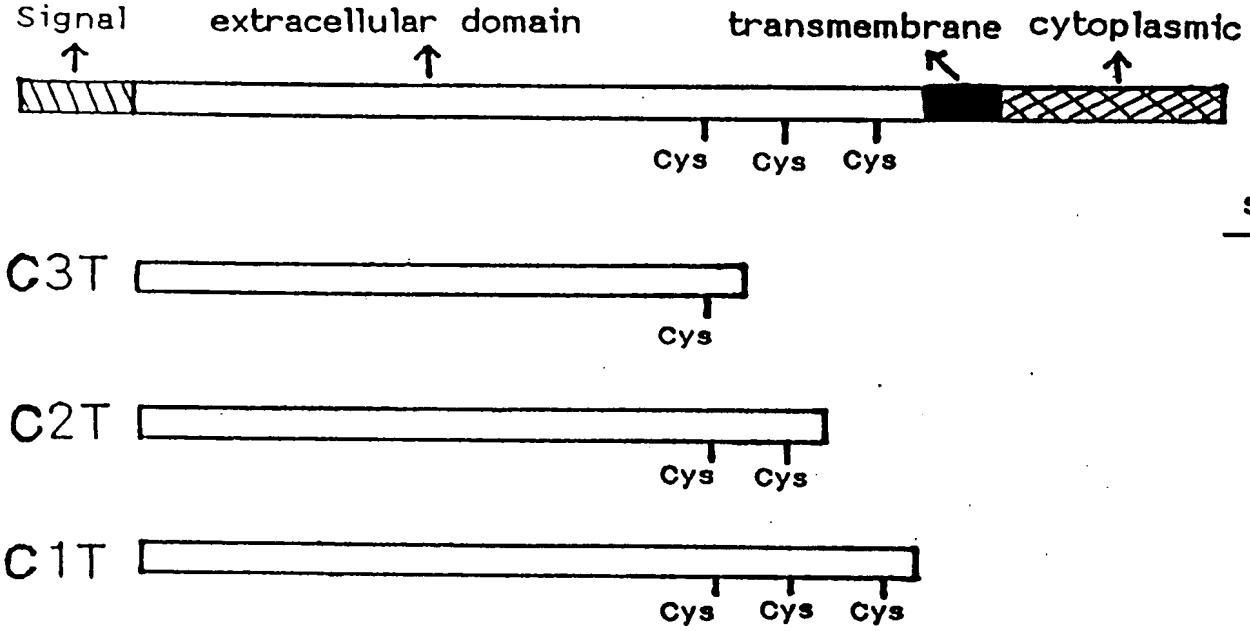
FIG. 4

```
AGA CTC TCA GGC CTT GGC AGG TGC GTC TTT CAG TTC CCC TCA CAC TTC   48

GGG TTC CTC GGG GAG GAG GGG CTG GAA CCC TAG CCC ATC GTC AGG ACA   96

AAG ATG CTC AGG CTG CTC TTG GCT CTC AAC TTA TTC CCT TCA ATT CAA  144
     M   L   R   L   L   L   A   L   N   L   F   P   S   I   Q   15

GTA ACA GGA AAC AAG ATT TTG GTG AAG CAG TCG CCC ATG CTT GTA GCG  192
 V   T   G   N   K   I   L   V   K   Q   S   P   M   L   V   A   31

TAC GAC AAT GCG GTC AAC CTT AGC TGC AAG TAT TCC TAC AAT CTC TTC  240
 Y   D   N   A   V   N   L   S   C   K   Y   S   Y   N   L   F   47

TCA AGG GAG TTC CGG GCA TCC CTT CAC AAA GGA CTG GAT AGT GCT GTG  288
 S   R   E   F   R   A   S   L   H   K   G   L   D   S   A   V   63

GAA GTC TGT GTT GTA TAT GGG AAT TAC TCC CAG CAG CTT CAG GTT TAC  336
 E   V   C   V   V   Y   G   N   Y   S   Q   Q   L   Q   V   Y   79

TCA AAA ACG GGG TTC AAC TGT GAT GGG AAA TTG GGC AAT GAA TCA GTG  384
 S   K   T   G   F   N   C   D   G   K   L   G   N   E   S   V   95

ACA TTC TAC CTC CAG AAT TTG TAT GTT AAC CAA ACA GAT ATT TAC TTC  432
 T   F   Y   L   Q   N   L   Y   V   N   Q   T   D   I   Y   F  111
```

FIG. 4 CONT.

```
TGC AAA ATT GAA GTT ATG TAT CCT CCT CCT TAC CTA GAC AAT GAG AAG   480
 C   K   I   E   V   M   Y   P   P   P   Y   L   D   N   E   K   127

AGC AAT GGA ACC ATT ATC CAT GTG AAA GGG AAA CAC CTT TGT CCA AGT   528
 S   N   G   T   I   I   H   V   K   G   K   H   L   C   P   S   143

CCC CTA TTT CCC GGA CCT TCT AAG CCC TTT TGG GTG CTG GTG GTG GTT   576
 P   L   F   P   G   P   S   K   P   F   W   V   L   V   V   V   159

GGT GGA GTC CTG GCT TGC TAT AGC TTG CTA GTA ACA GTG GCC TTT ATT   624
 G   G   V   L   A   C   Y   S   L   L   V   T   V   A   F   I   175

ATT TTC TGG GTG AGG AGT AAG AGG AGC AGG CTC CTG CAC AGT GAC TAC   672
 I   F   W   V   R   S   K   R   S   R   L   L   H   S   D   Y   191

ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG   720
 M   N   M   T   P   R   R   P   G   P   T   R   K   H   Y   Q   207

CCC TAT GCC CCA CCA CGC GAC TTC GCA GCC TAT CGC TCC TGA           762
 P   Y   A   P   P   R   D   F   A   A   Y   R   S   *           221
```

FIG. 5



	soluble	dimeric	the induction of T cell anergy
C3T	yes	no	no
C2T	yes	no	no
C1T	yes	yes	yes

FIG. 6

A. sequences of primers used in PCR reaction:

Primer	Sequence (5' ⟶ 3')	Direction
P1	CCTCGAGCATGCTCAGGCTGCTCTTG	forward
P2	TCTCTAGACTTAGAACCTCCGGGAAA	reverse
P3	AGTCTAGATGCCTGGAGCCCTACACC	forward
P4	TTATCAGGGAGCAGTGGCCGTCTC	reverse

FIG. 6 CONT.

B. Sequences at the junction between C1T and PAP

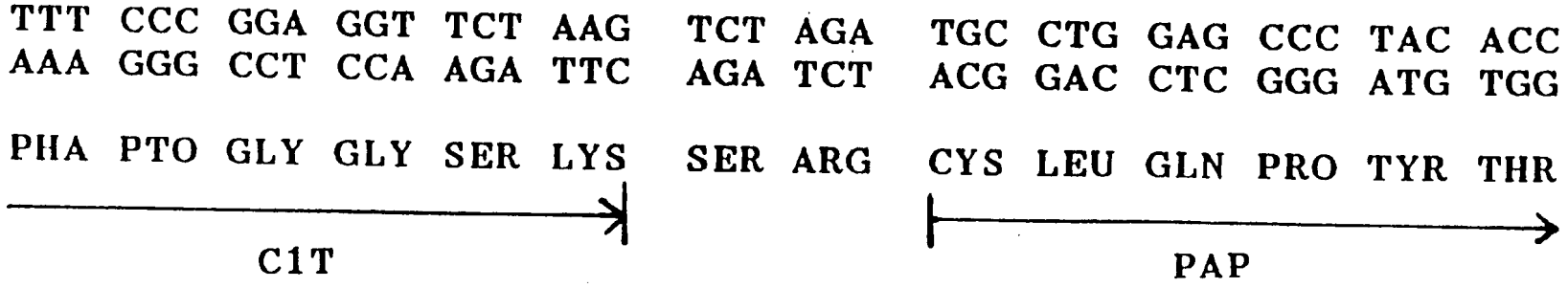


FIG. 7

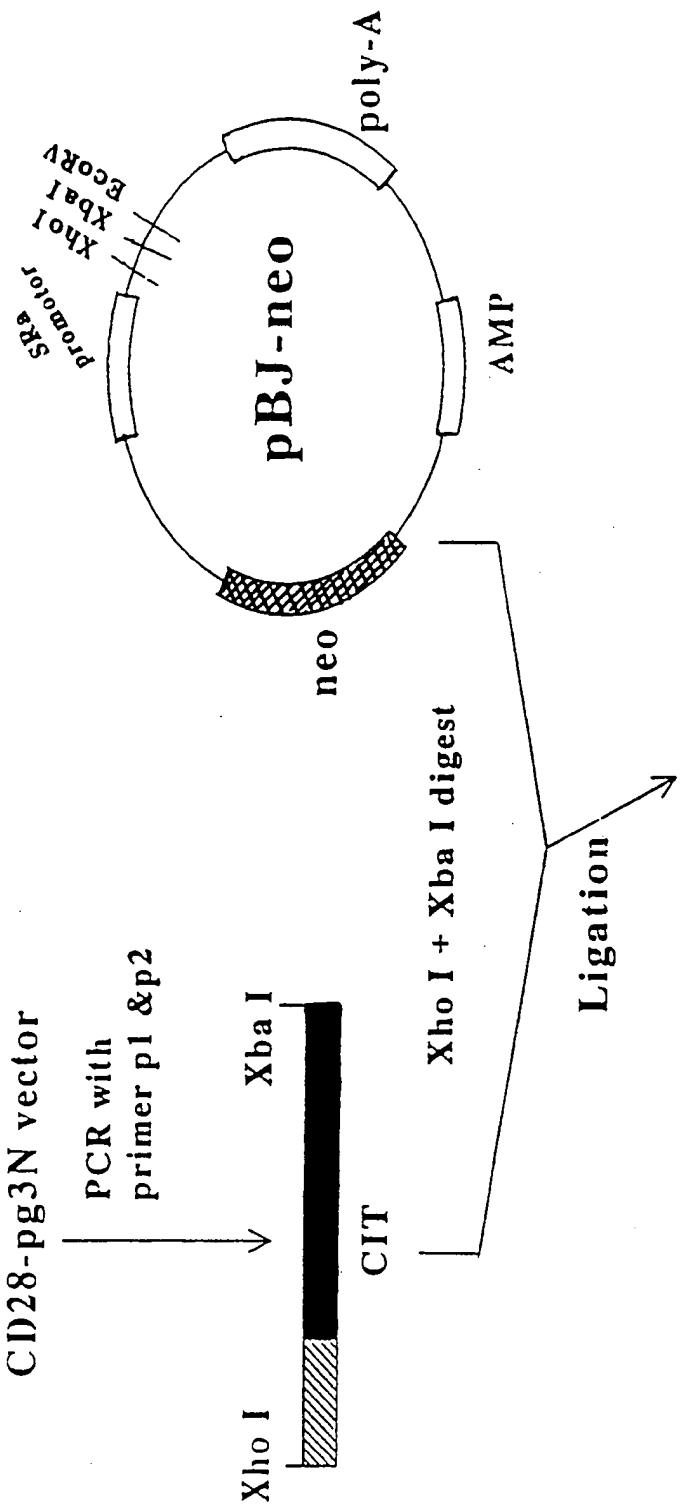
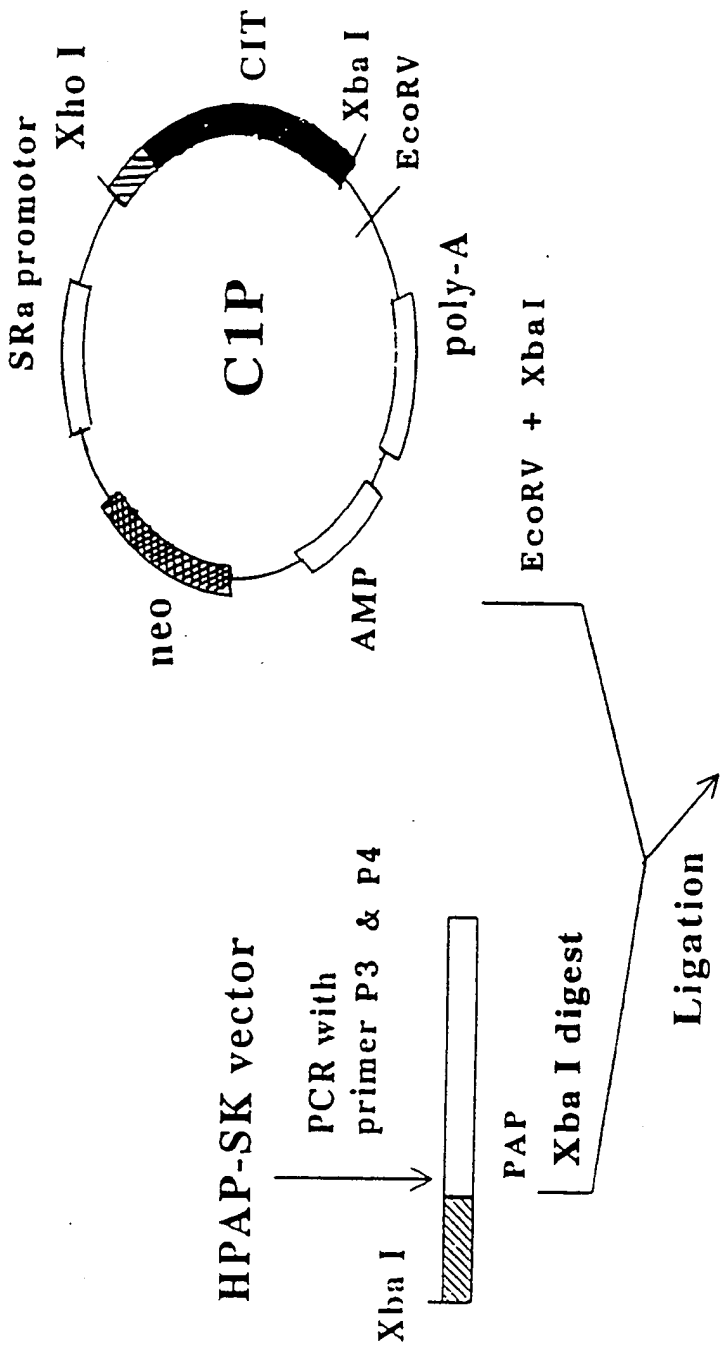

CD28-pg3N vector
PCR with
primer p1 &p2
Xba I
Xho I
CIT
Xho I + Xba I digest
Ligation
pBJ-neo
poly-A
AMP
neo
SRα promotor
Xho I
Xba I
EcoRV

FIG. 7 CONT.


SRa promotor
Xho I
CIT
Xba I
EcoRV
poly-A
C1P
neo
AMP
EcoRV + XbaI
HPAP-SK vector
PCR with
primer P3 & P4
Xba I
PAP
Xba I digest
Ligation

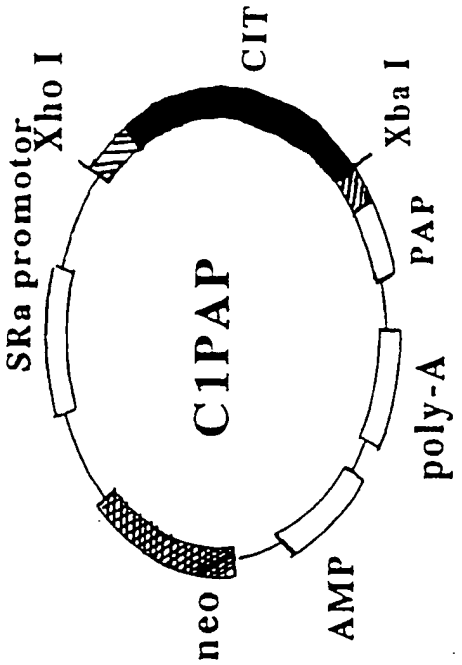


FIG. 7 CONT.

FIG. 8

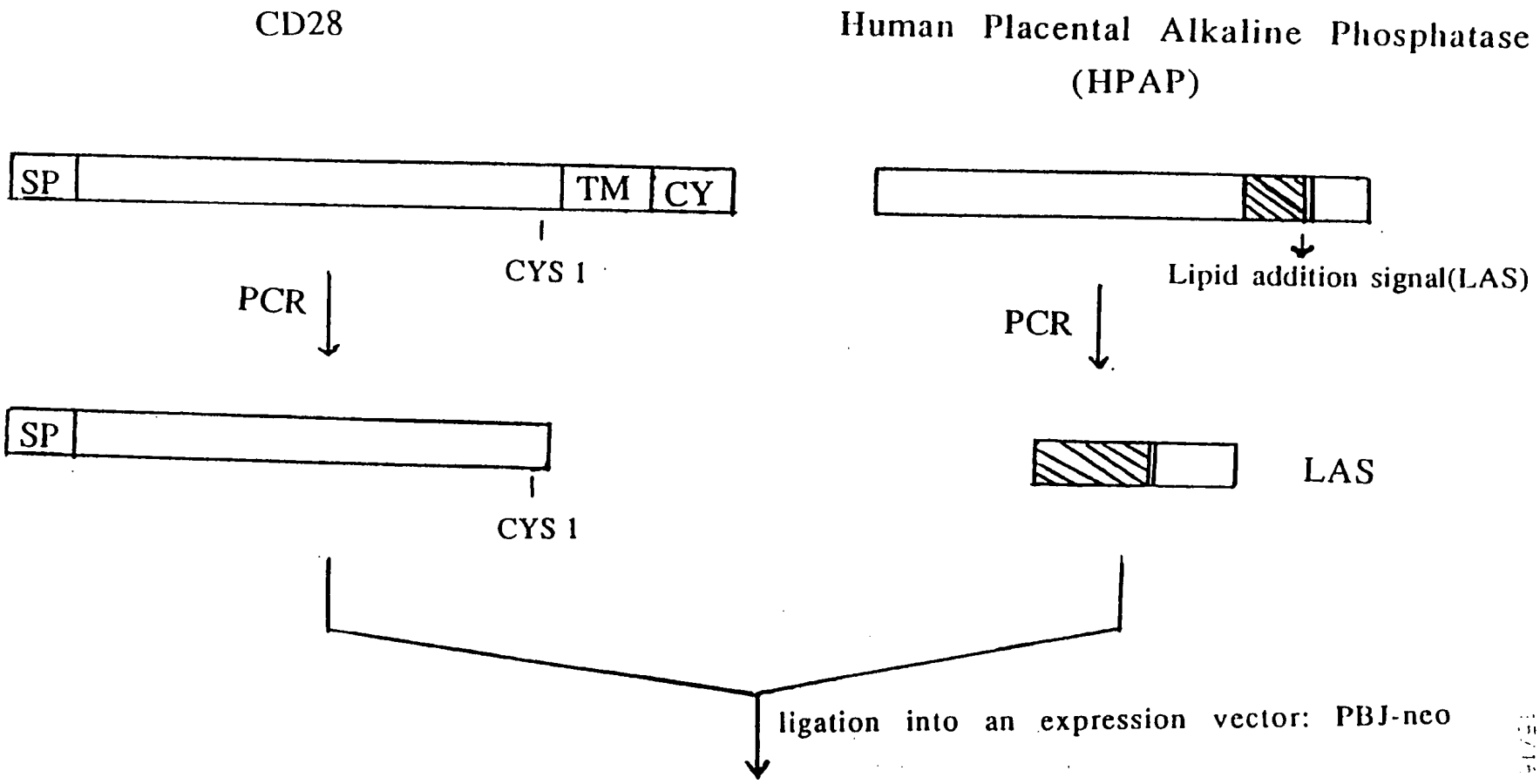
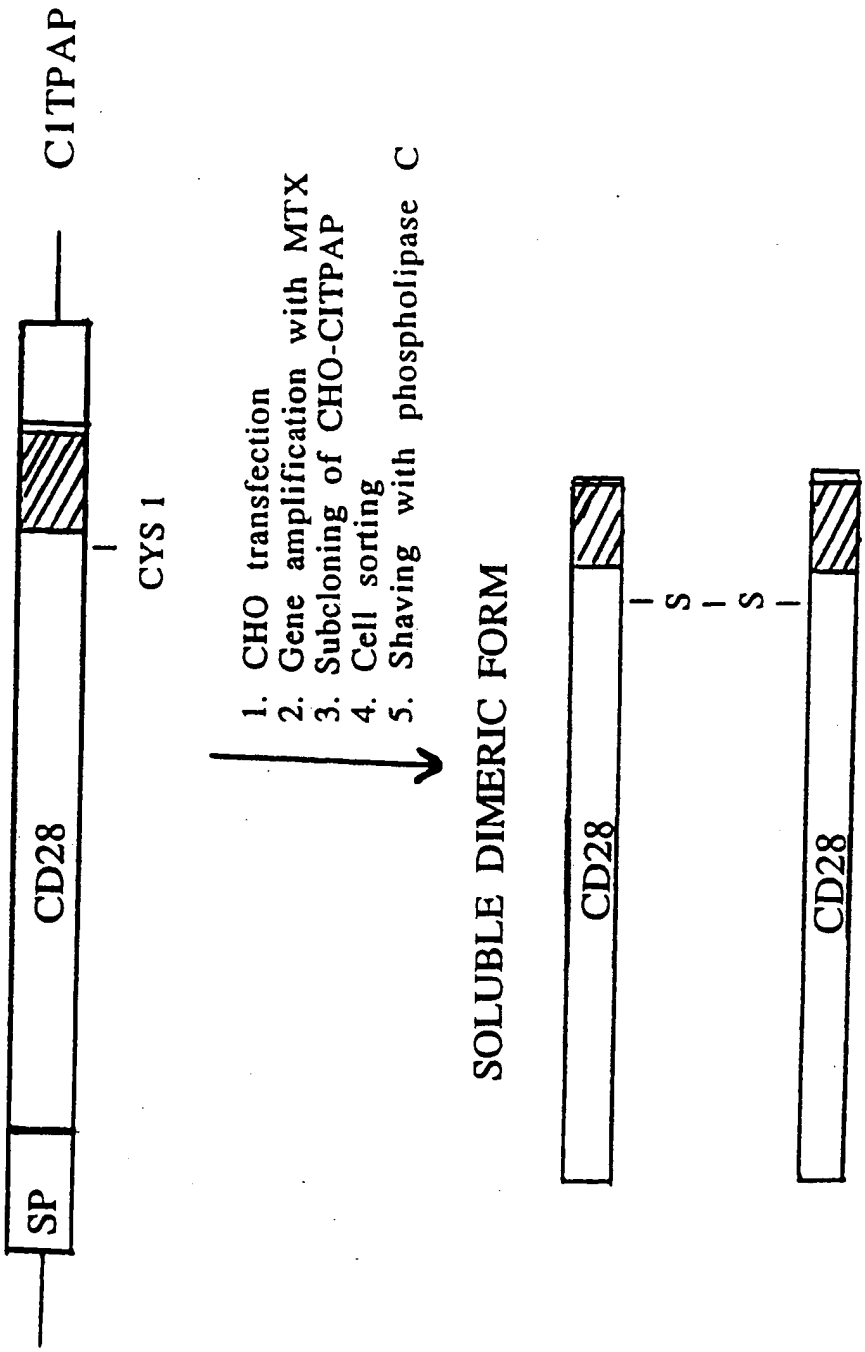

CD28
Human Placental Alkaline Phosphatase
(HPAP)
SP
TM
CY
CYS 1
PCR
Lipid addition signal(LAS)
PCR
SP
CYS 1
LAS
ligation into an expression vector: PBJ-neo

FIG. 8 CONT.


SP
CD28
CYS 1
CITPAP
1. CHO transfection
2. Gene amplification with MTX
3. Subcloning of CHO-CITPAP
4. Cell sorting
5. Shaving with phospholipase C
SOLUBLE DIMERIC FORM
CD28
S
S
CD28

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01867

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 5/00
US CL : 435/240.2

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/240.2

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS, Dialog, Intelligenetics

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	J. Exp. Med., Vol. 173, issued March 1991, P.S. Linsley et al., "Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation", pages 721-730, see entire document.	1-3,15-21/4-14
Y	Proc. Natl. Acad. Sci., USA, Vol. 87, issued July 1990, P.S. Linsley et al., "T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1", pages 5031-5035, see entire document.	1-21
X/Y	Proc. Natl. Acad. Sci., USA, Vol. 84, issued December 1987, A. Aruffo & B. Seed, "Molecular cloning of a CD28 cDNA by a high-effeiciency COS cell expression system", pages 8573-8577, see entire document.	15-21/1-14

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
16 June 1992	26 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	KAREN COCHRANE CARLSON, PH.D.